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Early Maturation of Gonadotropin-Releasing Hormone Secretion and Sexual Precocity after Exposure of Infant Female Rats to Estradiol or Dichlorodiphenyltrichloroethane[1]

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ABSTRACT

An increase in the frequency of pulsatile gonadotropin-releasing hormone (GnRH) secretion in vitro and a reduction in LH response to GnRH in vivo characterize hypothalamic-pituitary maturation before puberty in the female rat. In girls migrating for international adoption, sexual precocity is frequent and could implicate former exposure to the insecticide dichlorodiphenyltrichloroethane (DDT), since a long-lasting DDT derivative has been detected in the serum of such children. We aimed at studying the effects of early transient exposure to estradiol (E_2) or DDT in vitro and in vivo in the infantile female rat. Using a static incubation system of hypothalamic explants from 15-day-old female rats, a concentration- and time-dependent reduction in GnRH interpulse interval (IPI) was seen during incubation with E_2 and DDT isomers. These effects were prevented by antagonists of alpha-amino-3-hydroxy-5-methyl-isoxazole-4 propionic acid (AMPA)/kainate receptors and estrogen receptor. Also, o,p′-DDT effects were prevented by an antagonist of the aryl hydrocarbon orphan dioxin receptor (AHR). After subcutaneous injections of E_2 or o,p′-DDT between Postnatal Days (PNDs) 6 and 10, a decreased GnRH IPI was observed on PND 15 as an ex vivo effect. After DDT administration, serum LH levels in response to GnRH were not different from controls on PND 15, whereas they tended to be lower on PND 22. Subsequently, early vaginal opening (VO) and first estrus were observed together with a premature age-related decrease in LH response to GnRH. After prolonged exposure to E_2 between PNDs 6 and 40, VO occurred at an earlier age, but first estrus was delayed. We conclude that a transient exposure to E_2 or o,p′-DDT in early postnatal life is followed by early maturation of pulsatile GnRH secretion and, subsequently, early developmental reduction of LH response to GnRH that are possible mechanisms of the subsequent sexual precocity. The early maturation of pulsatile GnRH secretion could involve effects mediated through estrogen receptor and/or AHR as well as AMPA/kainate subtype of glutamate receptors.

environment, estradiol, gonadotropin-releasing hormone, hypothalamus, puberty

INTRODUCTION

During the past decade, precocious puberty (PP) was reported to occur frequently in foreign girls after migration for adoption in different countries of Western Europe. Among the possible pathophysiologic mechanisms, we have hypothesized that migration could result in withdrawal from exposure to estrogenic endocrine-disrupting chemicals (EDCs) in the home country and cause sexual precocity to occur following early hypothalamic maturation caused by the EDCs [1, 2]. Those environmental substances are known to interact with the reproductive system in a harmful manner [3, 4]. The involvement of EDCs in sexual precocity in migrating girls was suggested based on the detection of p,p′-dichlorodiphenyldichloroethene (p,p′-DDE) in the serum of those patients [1]. With a half-life of several decades, p,p′-DDE is a persistent derivative of the insecticide dichlorodiphenyltrichloroethane (DDT), which has been banned in the United States of America and Western European countries since the late 1960s [5, 6] but is still used extensively in developing countries [2]. This EDC is known to act as an estrogen receptor (ER) agonist and/or androgen receptor antagonist, both in vitro and in vivo [7, 8]. In the above situation, because p,p′-DDE levels were related directly to the length of stay in the country of origin and inversely to time since immigration [2], it was thought to be a marker of previous exposure to DDT during early life.

In immature animals, direct effects on peripheral tissues, such as the vaginal epithelium, were reported after exposure to estradiol (E_2) or estrogenic EDCs and were consistent with peripheral sexual precocity [7–9]. In 1971, Heinrichs et al. reported that administration of 1 mg o,p′-DDT in neonatal female rats on Postnatal Days (PNDs) 2–4 resulted in earlier vaginal opening (VO) and first estrus and delayed anovulatory syndrome [10]. They hypothesized that exposure to DDT in early life could cause premature hypothalamic-pituitary maturation and disturb the hypothalamic control of ovulation through unknown mechanisms. Since the o,p′-isomer of DDT had estrogenic uterotrophic properties [11], the question arose as to whether central PP could coexist or follow peripheral PP after exposure to DDT [2, 9]. A hypothalamic effect of estrogenic substances in the female individual was supported by our previous observation that E_2 preferentially stimulated gonadotropin-releasing hormone (GnRH) pulse frequency in the immature female rat hypothalamus in vitro through a mechanism dependent on the perinatal sexual differentiation. In addition, a single massive dose of E_2 given on PND 10 caused early maturation of pulsatile GnRH secretion, with early VO and first estrus subsequently [12]. The aim of the present study was to investigate, both in vitro and in vivo, the effects of an early transient exposure of the immature female rat hypothalamus and pituitary gland to DDT in comparison with E_2 and the mechanisms involved in such effects. Two

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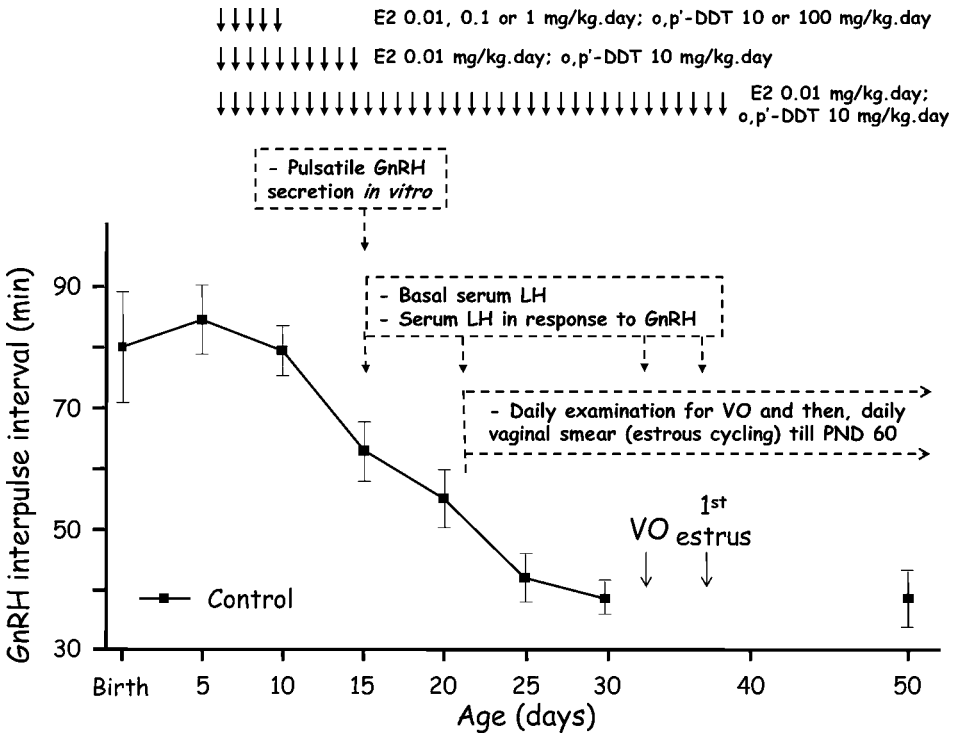

FIG. 1. Experimental design to study in vivo the effects of E_2 or o,p′-DDT treatment started in 6-day-old female rats as daily s.c. injections and maintained for 5, 10, or 35 days. The procedure is represented in relation to age with reference to the developmental reduction in GnRH IPI observed in vitro (n = 6). The average ages at VO and first estrus are also represented.

particular endpoints were chosen based on developmental characteristics: the frequency of pulsatile GnRH secretion by hypothalamic explants in vitro that shows a prepubertal acceleration between PNDs 10 and 25, as illustrated in Figure 1 [13, 14], and the LH secretory response to a synthetic GnRH administration in vivo that shows a prepubertal reduction until PND 36 [15, 16].

MATERIALS AND METHODS

Animals

Infantile female Wistar rats were purchased from the University of Liège. They were housed in standardized conditions with lactating dams (22°C, lights on from 0630 to 1830 h, food and water ad libitum). Each litter contained 5–10 pups. The day of birth was considered PND 1. The weaning occurred on PND 21. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège.

Hypothalamic Explant Incubation and GnRH Assay

The developmental variations in GnRH pulse frequency in vitro were studied using hypothalamic explants obtained in female rats on PNDs 1, 5, 10, 15, 20, 25, 30, and 50. For the in vitro study of hypothalamic explants, 15-day-old animals were used. After decapitation, the hypothalamus was rapidly dissected. The limits to obtain the retrochiasmatic hypothalamus were the caudal margin of the optic chiasm, the caudal margin of the mammillary bodies, and the lateral hypothalamic sulci [14]. Each explant was transferred into an individual chamber in a static incubator, as described in detail previously [12, 14]. Each chamber contained 500 μl minimum essential medium (MEM) supplemented with glucose, magnesium, glycine, and bacitracin to achieve final concentrations of 25×10^{-3}, 10^{-3}, 10^{-8}, and 2×10^{-5} M, respectively. The explants were incubated in an atmosphere of 95% O_2/5% CO_2 for a total period varying between 4 and 6 h. The incubation medium was collected and renewed every 7.5 min and was kept frozen until assayed.

The GnRH release in the incubation medium of hypothalamic explants was measured in duplicate using a radioimmunoassay method with intraassay and interassay coefficients of variation of 14% and 18%, respectively [17, 18]. The highly specific CR11-B81 anti-GnRH antiserum (final dilution 1:80 000) was kindly provided by Dr. V. D. Ramirez (Urbana, IL) [19]. The data below the limit of detection (5 pg/7.5-min fraction) were assigned that value.

GnRH Stimulation Test and LH Assay

At PNDs 15 and 22, and at VO and first estrus, serum LH levels were measured in basal conditions (s.c. injection of vehicle or o,p′-DDT) and 30 min after stimulation through s.c. injection of 1 μg/kg GnRH. These conditions were used after testing different time points (15, 30, and 45 min) following GnRH at different ages (15 and 20 days and at VO) and were consistent with the conditions reported by others [16, 20].

After 2 h of clotting at room temperature, trunk blood collected from the killed animals was centrifuged (5 min at $2000 \times g$). The serum was collected and stored at −20°C until assayed. Serum LH levels were determined in duplicate in a volume of 100 μl using a double-antibody method and radioimmunoassay kits kindly supplied by the National Institutes of Health (Dr. A.F. Parlow, National Institute of Diabetes and Digestive & Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-8 was labeled with ^{125}I by the chloramine-T method. The hormone concentrations were expressed using the reference preparation rLH-RP-3 as a standard. The intraassay and interassay coefficients of variation were 7% and 9%, respectively. The sensitivity limit of the assay was 5 ng/ml.

Reagents

The MEM was purchased from Life Technologies Invitrogen Corp. (Merelbeke, Belgium). E_2 (17β-estradiol or 3,17β-dihydroxy-1,3,5(10)-estratriene); the two DDT isomers, o,p′-DDT (2,4′-DDT) and p,p′-DDT (4,4′-DDT); and p,p′-DDE (4,4′-DDE) were purchased from Sigma-Aldrich (Bornem, Belgium). P,p′-DDT represents approximately 80% of the insecticide still commonly used in developing countries. O,p′-DDT is an equally active isomer of the insecticide that accounts for 15%–20% of technical grade DDT. In endocrine studies, o,p′-DDT has been particularly studied due to its prominent estrogenic property and relatively less toxic activity. P,p′-DDE is a long-lasting derivative of p,p′-DDT, with a half-life of several years. The α-amino-3-hydroxy-5-methylisoxazole-4 propionic acid (AMPA)/kainate subtype of glutamate receptor antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione) and the ER antagonist ICI 182 780 (7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-1,3,5(10)-estratriene-3,17β-diol) were purchased from Tocris Fisher Bioblock Scientific (Illkirch, France), whereas the aryl hydrocarbon orphan dioxin receptor (AHR) antagonist α-naphtoflavone (7,8-benzoflavone) was purchased from Sigma-Aldrich. In all experiments, the steroid and insecticides were dissolved initially in absolute ethanol (Labonord, Templenars, Belgium) and, subsequently, in the incubation medium or sesame oil (Calbiochem VWR International, Leuven, Belgium) for in vitro or in vivo studies, respectively, to

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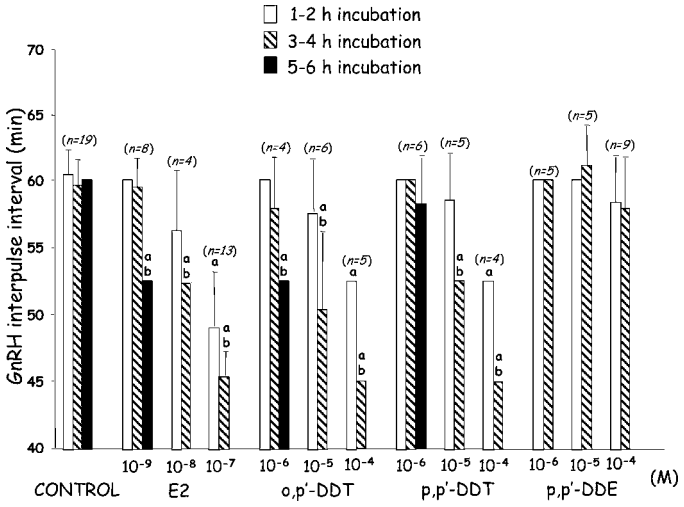

FIG. 2. Effects of E_2, DDT isomers, and p,p′-DDE in vitro on the frequency of pulsatile GnRH secretion from hypothalamic explants obtained in 15-day-old female rats. The data are calculated in relation to time (two or three consecutive 2-h periods) of incubation in vitro. a: $P < 0.05$ versus control; b: $P < 0.05$ versus data obtained during the initial 2-h period.

achieve a final ethanol concentration of 0.01% or 1%. The antagonists were directly diluted in the incubation medium.

Study Protocols

In vitro experiments. The in vitro effects of E_2 or DDT on the frequency of pulsatile GnRH secretion were studied. The compounds (steroid, DDT isomers, and antagonists) were used for a whole 4- or 6-h experimental incubation period. A concentration-response study was carried out with explants incubated with 10^{-9} to 10^{-7} M of E_2 and 10^{-6} to 10^{-4} M of o,p′-DDT, p,p′-DDT, or p,p′-DDE. The effects of antagonists were studied in the presence of maximal effective concentrations of E_2 and o,p′-DDT. Those antagonists were chosen because they were shown to prevent E_2 effects on GnRH secretion in our hypothalamic explant conditions [12, 21], and it was our hypothesis that DDT effects, if any, could be mediated through mechanisms similar to E_2 effects. The antagonist DNQX (10^{-6} M) was used to study the involvement of the AMPA/kainate subtype of glutamate receptors. The implication of ER was studied using the antagonist ICI 182 780 (10^{-7} M). To investigate the implication of AHR, the antagonist α-naphtoflavone (10^{-7} M) was used. The concentration of these three antagonists was selected based on previous data from our laboratory and other studies [12, 17, 21–23]. It was shown previously that when used alone, the AMPA/kainate subtype of glutamate receptor and ER antagonists did not affect pulsatile GnRH secretion [12], and the absence of effects of the different antagonists when used alone was double checked in this study.

In vivo experiments. The procedures are schematically summarized in Figure 1. The animals received a daily s.c. administration of steroid or insecticide for 5, 10, or 35 days (E_2: 0.01, 0.1, or 1 mg/kg/day for PNDs 6–10 and 0.01 mg/kg/day for PNDs 6–15 and PND 6–40; o,p′-DDT: 10 or 100 mg/kg/day for PNDs 6–10 and 10 mg/kg/day for PNDs 6–15). The dose of E_2 and o,p′-DDT was adjusted for increasing body weight of rats. The chemicals dissolved in absolute ethanol were diluted in 50 μl sesame oil for s.c. injection, as described in other studies [24, 25]. When the treatment period was PNDs 6–10, 20 rats were studied in each treated group in comparison with 20 controls injected with vehicle alone. On PND 15, 10 rats from each group were killed to study the pulsatile GnRH secretion in vitro and serum LH levels. In each group, the 10 remaining animals were then examined daily for imperforation of the vaginal membrane to determine age at VO. Thereafter, vaginal smears were taken every day in the afternoon until PND 60. Slides of vaginal smears were colored using the Papanicolaou method to detect the occurrence of estrous cyclicity and to follow cycling. The age at first estrus was considered when vaginal smears contained primary cornified cells after the first proestrous phase, which is characterized by both stratified and cornified cells [26]. In subsequent experiments to study LH response to GnRH on PNDs 15 and 22, and at the time of VO and first estrus, seven animals were killed in each group at each age. When the treatment period was PNDs 6–15, there were five rats in each group that were killed on PND 15 to study the GnRH pulse frequency and serum LH levels. When the treatment period was PNDs 6–40, there were 10 rats followed in each condition to study VO and estrous cyclicity.

Statistical Analysis

When pulsatile GnRH secretion was studied, the peaks were detected using the PULSAR program for PC [27]. The cutoff criteria for peak detection were determined empirically and were $G_1 = 2.5$ and $G_2 = 2.0$. Peak splitting parameter was set at 2.7, and intraassay coefficient of variation was used as B coefficient [28]. The GnRH interpulse interval (IPI) was calculated as the time interval between two consecutive peaks. The IPI was calculated during different time periods of incubation (1–2 h and 3–4 h or 5–6 h). Depending on the normal or nonnormal distribution of IPI data in the different study periods, comparisons were made using the paired Student *t*-test with $P < 0.05$ as the threshold for significance (GraphPad Prism software for PC) or the Wilcoxon matched pairs test, respectively. In several instances, all the explants in a group showed a similar IPI value. In this case, SD was null and could not be represented.

When comparisons were made between steroid and/or insecticide effects on LH levels and age at VO or first estrus in different conditions, raw data were pooled and analyzed by the one-way ANOVA test when normally distributed, followed by a multiple-comparison Newman-Keuls post-hoc test when the threshold for significance of differences ($P < 0.05$) was reached. When data were not normally distributed, the Kruskal-Wallis test was used, followed by a multiple-comparison Dunn post-hoc test. For the experiment run PNDs 6–40, an unpaired *t*-test was employed. All results are expressed as mean ± SD.

RESULTS

In Vitro Treatments

In control conditions of hypothalamic explant incubation in vitro (Fig. 1), the GnRH IPI showed a reduction between PNDs 10 and 25, confirming our data in the male [13]. When hypothalamic explants obtained at 15 days were incubated in control conditions (Fig. 2), the GnRH IPI did not change with time (1–2 h: 60.4 ± 1.7 min; 3–4 h: 59.6 ± 1.8 min; and 5–6 h: 60.0 ± 0.0 min). During a 4-h continuous incubation with 10^{-7} M of E_2, the GnRH IPI was reduced significantly after 1–2 h (49.0 ± 3.9 min) and further after 3–4 h (45.4 ± 1.7 min). This effect was dependent on E_2 concentration and incubation time: with 10^{-8} M E_2, the GnRH IPI was unchanged after 1–2 h and decreased significantly after 3–4 h; with 10^{-9} M E_2, a significant reduction occurred after 5–6 h only (52.5 ± 0.0 min). The two active isomers of DDT also caused a concentration- and time-dependent reduction in GnRH IPI that was significant after 3–4 h using 10^{-5} M of both isomers (o,p′-DDT: 50.4 ± 5.7 min; p,p′-DDT: 52.5 ± 0.0 min). At a 10^{-4} M concentration, both isomers resulted in an earlier effect that was also greater after 3–4 h (o,p′-DDT: 45.0 ± 0.0 min; p,p′-DDT: 52.5 ± 0.0 min). When used at 10^{-6} M, p,p′-DDT had no effect during a 6-h incubation, and o,p′-DDT showed a significant effect only after 5–6 h of incubation (52.5 ± 0.0 min). No effect could be obtained using p,p′-DDE at similar concentrations.

The significant reduction of GnRH IPI caused by 10^{-7} M E_2 or 10^{-4} M o,p′-DDT after 3–4 h of incubation in vitro (Fig. 3A) was totally prevented when the AMPA/kainate subtype of glutamate receptors was antagonized by coincubation with DNQX (Fig. 3B). Likewise, the effects of E_2 and o,p′-DDT were totally prevented in the presence of the ER antagonist ICI 182 780 (Fig. 3C). When α-naphtoflavone was used to antagonize the AHR, the significant decrease in GnRH IPI caused by o,p′-DDT was not observed any more, whereas the E_2 effect was attenuated but remained significant (Fig. 3D).

In Vivo Treatments

As shown in Figure 4A, after 5 days of treatment with 0.01, 0.1, and 1 mg/kg E_2 (PNDs 6–10), the age at VO (controls:

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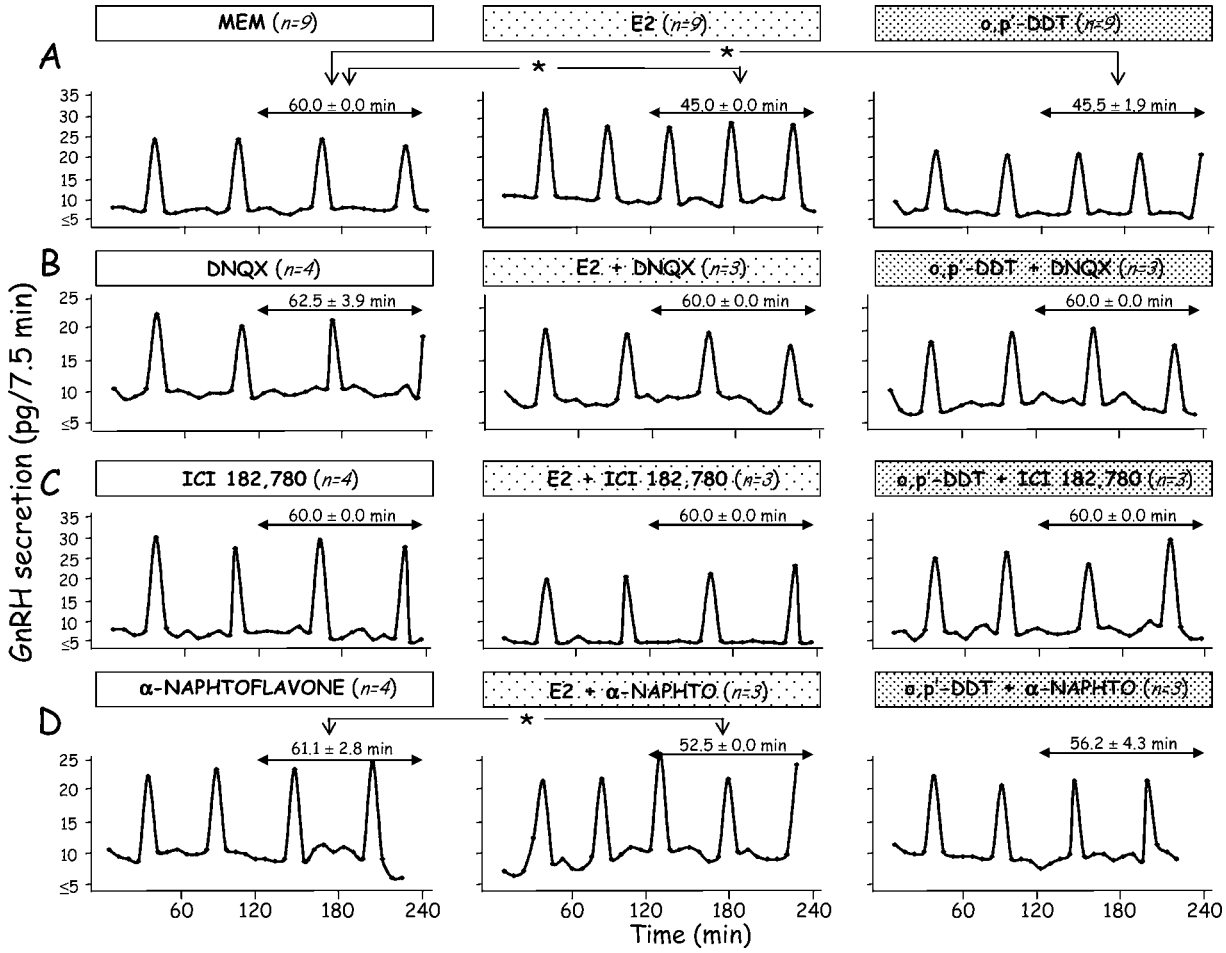

FIG. 3. Effects of 10^{-6} M DNQX, an antagonist of the AMPA/kainate subtype of glutamate receptors (**B**), 10^{-7} M of ICI 182 780, an ER antagonist (**C**), and 10^{-7} M α-naphtoflavone, an AHR antagonist (**D**) on the GnRH IPI during incubation of hypothalamic explants obtained in 15-day-old female rats in the presence of 10^{-7} M E_2 or 10^{-4} M o,p′-DDT (**A**) in vitro. A representative profile of pulsatile GnRH secretion is shown in each condition, and the mean (± SD) IPI observed during 3–4 h of incubation are given. *$P < 0.05$ treatment versus control conditions.

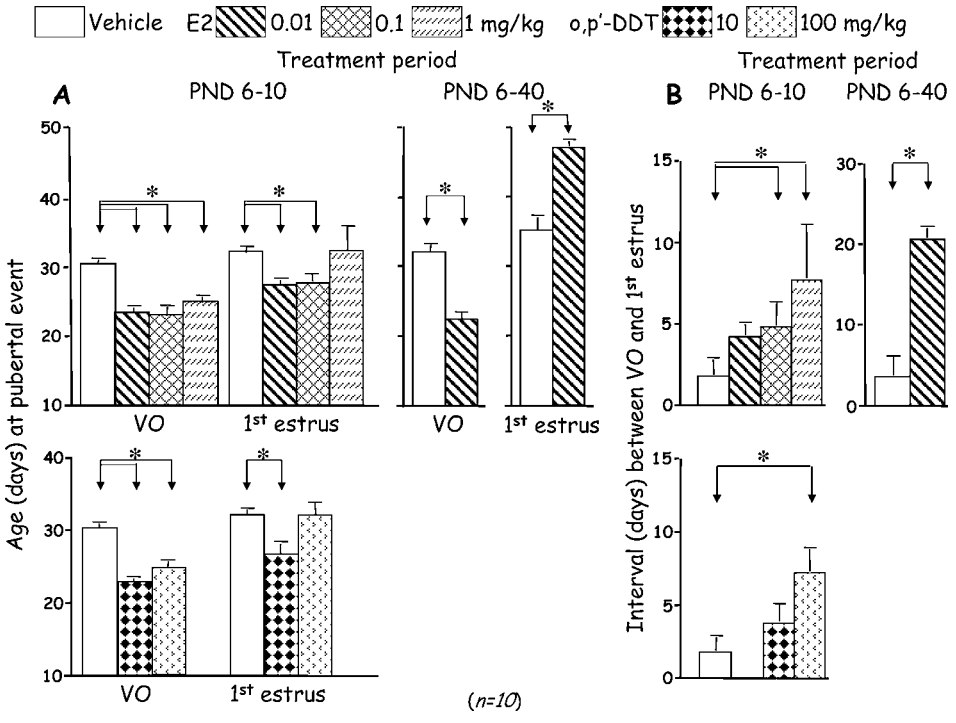

FIG. 4. Effects of E_2 or o,p′-DDT injected s.c. in female rats for PNDs 6–10 or PNDs 6–40 on the ages at VO and first estrus (**A**) and the interval between VO and first estrus (**B**). *$P < 0.05$ versus vehicle (sesame oil).

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TABLE 1. Mean ± SD (n = 10) of estrous cycle length (interval between two consecutive estrus) observed until PND 60.

Treatment	Dose (mg/kg)	Estrous cycle length (days)
PND 6–10		
Vehicle	—	4.7 ± 1.4
E2	0.01	5.2 ± 1.6
	0.10	5.5 ± 1.8
	1.00	4.6 ± 1.5
o,p′-DDT	10.00	5.3 ± 2.0
	100.00	5.5 ± 1.4
PND 6–40		
Vehicle	—	4.8 ± 1.2
E2	0.01	4.9 ± 1.8

30.1 ± 0.6 days) was significantly earlier (23.0 ± 0.7, 22.7 ± 1.3, and 24.6 ± 0.5 days, respectively). The VO was also earlier after 10 and 100 mg/kg o,p′-DDT (22.6 ± 0.5 and 24.6 ± 0.7 days, respectively). The first estrus was observed on PND 31.9 ± 0.7 in controls and occurred significantly earlier after 0.01 and 0.1 mg/kg of E_2 (27.2 ± 0.7 and 27.5 ± 1.1 days), as well as after 10 mg/kg o,p′-DDT (26.4 ± 1.5 days). After 1 mg/kg E_2 or 100 mg/kg o,p′-DDT, the age at first estrus did not change. When the time interval between VO and first estrus (Fig. 4B) was calculated (controls: 1.8 ± 0.9 days), a significant dose-dependent increase was observed after 0.1 and 1 mg/kg E_2 or 100 mg/kg o,p′-DDT. No differences in estrus cycle length were observed until PND 60 (Table 1).

As shown in Figure 5A, after five daily s.c. injections of 0.01, 0.1, and 1 mg/kg E_2 (PNDs 6–10), the GnRH IPI studied ex vivo on PND 15 (controls: 60.0 ± 0.0 min) was significantly reduced (54.4 ± 3.3, 52.5 ± 0.0, and 47.0 ± 3.4 min, respectively). A reduction of GnRH IPI was also seen after treatment with 10 and 100 mg/kg/day o,p′-DDT, which was only significant with 100 mg/kg (55.0 ± 3.6 min). When 0.01 mg/kg E_2 or 10 mg/kg o,p′-DDT was administered for a longer period of 10 days (PNDs 6–15), the GnRH IPI ex vivo was significantly reduced on PND 15 as well.

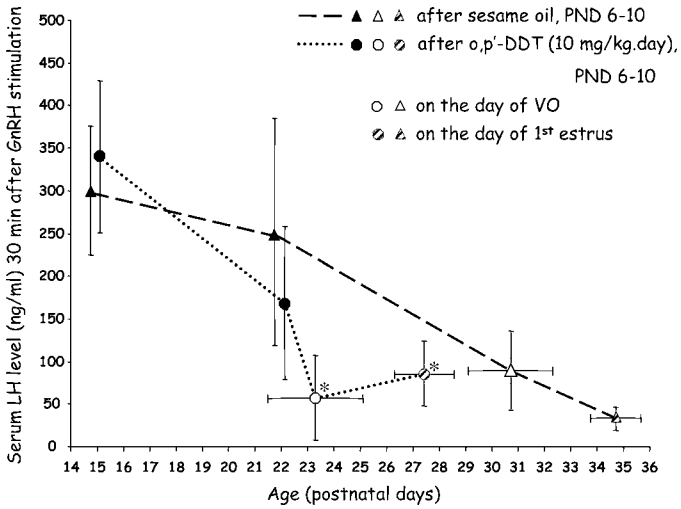

FIG. 6. Effects of treatment of female rats with o,p′-DDT (10 mg/kg/day) or sesame oil for PNDs 6–10 on serum LH levels studied 30 min after subcutaneous injection of 1 μg/kg GnRH at four age points: PND 15, PND 22, the day of VO, and the day of first estrus. The data are mean ± SD of LH levels and age in seven rats studied in each group. *$P < 0.05$ versus vehicle (sesame oil).

As shown in Figure 5B, the serum LH level on PND 15 (controls: 41.1 ± 19.9 ng/ml) was significantly decreased after 0.01, 0.1, and 1 mg/kg E_2 between PNDs 6 and 10 (18.9 ± 12.7, 23.7 ± 13.8, and 9.7 ± 3.0 ng/ml, respectively) and after 10 and 100 mg/kg o,p′-DDT (22.7 ± 18.5 and 15.7 ± 6.0 ng/ml, respectively). However, the serum LH level also decreased with age after sesame oil (control) administration (Fig. 6): 48.2 ± 46.7 ng/ml on PND 15, 25.8 ± 19.3 on PND 22, 16.5 ± 13.7 on PND 30.6 ± 1.5 (at VO), and 10.5 ± 7.3 on PND 35.0

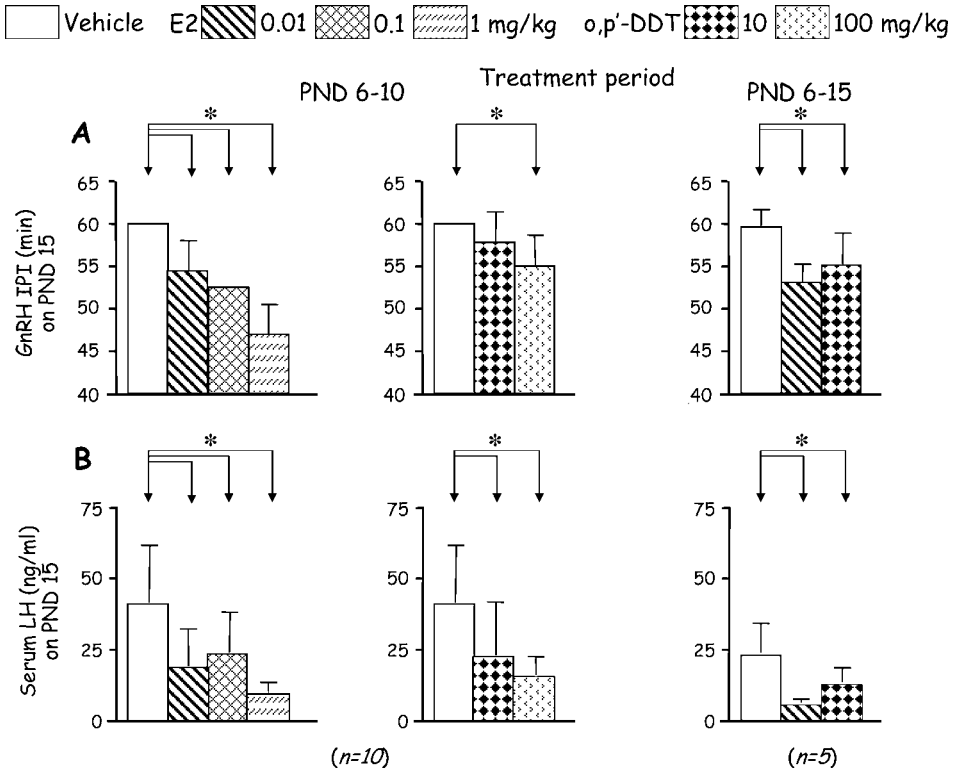

FIG. 5. Effects of E_2 or o,p′-DDT injected s.c. in female rats for PNDs 6–10 or PNDs 6–15 on the GnRH IPI (**A**) and serum LH concentrations (**B**) studied ex vivo at 15 days of age. *$P < 0.05$ versus vehicle (sesame oil).

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± 1.2 (at first estrus). When the LH response to GnRH was studied after vehicle or 10 mg/kg o,p′-DDT given for PNDs 6–10, the LH response was not affected on PND 15 and showed some but not significant reduction on PND 22, whereas it dropped significantly in the next days, at the time of early VO and first estrus.

An extended period of daily s.c. administration of 0.01 mg/kg E_2 for PNDs 6–40 (Fig. 4A) caused a significantly earlier age at VO (22.1 ± 1.0 vs. 31.8 ± 1.0 days in controls), whereas the age at first estrus was significantly delayed (47.1 ± 0.8 vs. 35.4 ± 1.7 days in controls), and the interval between VO and first estrus was markedly increased (20.6 ± 1.2 vs. 3.6 ± 2.2 days in controls). The first estrus was observed after a permanent estrus lasting until a few days after the end of treatment. Then, no differences in estrous cycle length were observed until PND 60 (Table 1). After daily s.c. administration of 10 mg/kg o,p′-DDT for PNDs 6–40, the animals showed altered health status with reduced activity and feeding. On PND 40, their weight was significantly decreased to 111.3 ± 8.3 g (130.0 ± 11.5 g after E_2 and 149.0 ± 9.9 g in controls). In such conditions, it was no longer possible to separate direct o,p′-DDT effects on sexual maturation and reproduction from indirect effects through disordered nutritional status.

DISCUSSION

The hypothalamic explant paradigm used in this study provided an opportunity to observe pulsatile GnRH secretion in vitro as the result of GnRH neuron function in its original surrounding neuronoglial apparatus, which regulates GnRH secretion [29]. Moreover, the developmental changes in frequency of pulsatile GnRH secretion retained in this model [13] made possible the study of interaction with maturational processes, although the critical age period for the developmental increase in GnRH pulse frequency was earlier in vitro [14] than in vivo [30, 31]. Such a difference could involve suppression of inhibitory extrahypothalamic inputs when explants are deafferented from the rest of the brain. However, the critical period of the second and third postnatal weeks in our conditions is consistent with neuroendocrine maturation preceding the peripheral changes of sexual maturation (VO, testicular growth). Since we incubated retrochiasmatic hypothalami containing prominently axons and terminals of GnRH-secreting neurons [32], we tend to interpret our observations as the effects of presynaptic regulation by the surrounding neurons and glial cells. Other in vitro paradigms involving GnRH neurons, such as GnRH-secreting neuronal cell lines [33–35], primary cultures of hypothalamic neurons [36–38], or hypothalamic slices from transgenic mice carrying reporter genes linked to the GnRH promoter [39] could enable one to study directly the regulatory mechanisms at the GnRH neuron level.

The present study was designed to test experimentally the hypothesis that early and transient exposure to pesticide, as seen in internationally adopted children, could influence hypothalamic-pituitary maturation and account for some central mechanism in the sexual precocity occurring frequently in such conditions. Since p,p′-DDE, which was detected in the serum of those children, was thought to result from previous exposure to DDT, this EDC was used for in vivo treatment. After we observed that o,p′-DDT and p,p′-DDT were almost equally effective on GnRH pulse frequency in vitro, the o,p′-DDT isomer was preferred for in vivo investigations based on its greater estrogenicity and lower toxicity than p,p′-DDT. In addition, o,p′-DDT was the isomer used in former studies on the neuroendocrine control of reproduction [10, 11, 40–42]. The doses that we used for in vivo studies and the E_2:o,p′-DDT concentration ratio were based on our in vitro data reported in the present study and were comparable with those used in other studies in rodents [43, 44]. The 10 and 100 mg/kg doses were in the same range as the amounts given by others either neonatally [10] or around the time of weaning [11]. After early postnatal administration, lower doses appeared to have no effects on age at VO and gonadotropin secretion [41]. It was not possible in this study to measure the concentrations of DDT isomers and derivatives in serum and tissues. The quantitative relevance of our conditions of exposure to DDT when compared to the exposure of migrating girls was difficult to assess, since the only available information was p,p′-DDE concentrations measured in serum several years later. After early DDT treatment in neonatal rats for 3 days, p,p′-DDE measured in adipose tissue 4–5 mo later was not different from controls, suggesting clearance of the insecticide and its residues after that long period [10]. After 7 days of daily oral intake of p,p′-DDT in a daily dose equivalent to 106 mg/kg in 5-wk-old rats, serum p,p′-DDE levels attained a mean level of 0.66 mg/l [45]. Such a serum concentration was about 20 times higher than the serum concentrations found in migrating children [1]. We, however, observed significant neuroendocrine effects using a 10-times-lower DDT dose that would presumably result in lower serum DDT levels. Further comparison would require measurement of the different DDT isomers and residues at different time points after stopping DDT treatment in the animals. The interpretation of doses and exposure is even more complex, since migrating children are likely to be exposed to various EDCs both in the original and foster countries. It has been reported that when chemicals are used in mixtures, combination effect could require lower concentrations than expected based on simply additive effects [46]. Moreover, when animals undergo low-dose exposure, the dose-effect relationship is not linear with comparatively greater effects of low versus high doses. Although these aspects have not been investigated in the present study, they could be important for the pathophysiologic relevance of our findings.

The age window of PNDs 6–10 could be consistent with early postnatal period in human infants. A 5-day period of treatment was relatively short for the lifespan but significant with respect to the short time period between birth and sexual maturation in the rat. The use of female rats aged 15 days to study GnRH IPI was based on our previous data showing that GnRH secretion in vitro was maximally affected by E_2 in such conditions [12]. With hypothalamic explants from younger females (5 days), GnRH secretion was also found to be responsive to E_2, but such age falls in the critical window of the brain sexual differentiation, causing possible interferences with programming of estrus cyclicity. This conclusion was drawn by Heinrichs et al., who found early VO and delayed persistent estrus to occur after DDT treatment for PNDs 2–4 [10]. Discrepant observations were made when o,p′-DDT treatment was started at 3 wk of age for several weeks: Gellert et al. found VO to occur earlier [11], whereas Wrenn et al. found no change in age at VO that was hastened only when treatment was given before the age of 3 wk [40]. In the present study, treatment during the second week of postnatal life was a compromise to possibly affect sexual maturation without interfering with the sexually differentiated mechanism of ovulation. However, such interferences probably did occur using the highest E_2 and DDT concentrations, since they did not result in early first estrus as opposed to lower doses. Alternatively, toxic effects could have occurred, although the nonsignificantly affected growth in those short treatment

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conditions did not support such an hypothesis. Three periods were chosen for in vivo exposure based on the attempt to mimic the either temporary or persisting exposure of children either migrating from or staying in developing countries. In the case of temporary exposure, two different lengths were studied (until PND 10 or 15) in order to investigate whether duration could influence the effect. This was confirmed since, after exposure for 10 instead of 5 days, the lowest dose of DDT became significantly effective in reducing the GnRH IPI, and the lowest dose of E_2 became more effective.

As many other EDCs, the DDT isomers were shown to exhibit both estrogenic and antiandrogenic properties, whereas p,p′-DDE retained only antiandrogenic activity [7, 8]. Because the clinical observation was made in girls [1], and E_2 was found to preferentially influence GnRH secretion in the female rat hypothalamus [12], female rats were used in the present study. In our experimental conditions, it was shown previously that both E_2 and testosterone could accelerate GnRH pulse frequency, the effect of androgens being aromatase dependent and ultimately mediated through estrogens [12]. Therefore, we hypothesized that the estrogenic activity of DDT was also involved in the pathogenesis of sexual precocity [1, 2, 9]. This hypothesis was consistent with our previous observation of sexual precocity after a single massive administration of E_2 on PND 10 [12], although the use of lower doses for a longer time period needed to be investigated. For all these reasons, E_2 was used as a positive control in this work to provide a comparison basis with DDT effects in vitro and in vivo.

Direct incubation of hypothalamic explants with E_2 or DDT isomers resulted in a concentration- and time-dependent increase in GnRH pulse frequency. The 1:1000 potency ratio of E_2:DDT found in our conditions was consistent with other studies [47]. P,p′-DDE had no effect, which is in agreement with the absence of estrogenlike effects reported in other conditions [48]. Although supraphysiologic concentrations of E_2 were required for an effect within 1–2 h, lower concentrations became effective after few hours of incubation. Such a delay could be explained by the slow diffusion of reagents into the explant, a hypothesis consistent with the observation that greater concentrations of compounds like excitatory amino acids are needed in explant paradigms compared with neuronal culture systems [49]. Another explanation could be the latency before the possibly genomic mechanisms involved in E_2 effects became effective. Then E_2 effects on GnRH secretion could have a rapid, presumably nongenomic component, as illustrated by Matagne et al. [21] in our paradigm, together with a slow genomic component. In this case, the target would be other cells than GnRH neurons, since GnRH cell bodies are absent from the studied retrochiasmatic explants [32]. Herbison [50] and, more recently, Herbison and Pape [51] have also reported that E_2 exerts complex effects on the GnRH neuronal function, including long-term or genomic effects through binding to ERα and/or ERβ subtypes. In our system, the acceleration of pulsatile GnRH secretion caused by E_2 was prevented by ICI 182 780, an α/β ER antagonist, as well as by DNQX, an antagonist of the AMPA/kainate subtype of glutamate receptors, confirming our previous observations [12]. The involvement of AMPA/kainate subtypes of glutamate receptors in E_2 stimulation of GnRH pulse frequency was further supported by the hypothalamic colocalization of those receptors together with ER [52]. Since the effects of o,p′-DDT were also prevented by ICI 182 780 and DNQX, this EDC appeared to involve the same receptors as E_2. However, the receptor pathway involved in o,p′-DDT effects could be partly different, with a participation of the orphan dioxin AHR, as indicated by the preferential reduction of o,p′-DDT effects by the antagonist α-naphtoflavone. The investigation of the insecticide effect through this pathway was justified, since Ohtake et al. [23] reported a few years ago that dioxins can mimic the effect of estrogens via a mechanism that involves the activation of ER by the transcriptionally active AHR-aryl hydrocarbon nuclear translocator complex. Further studies could delineate the relative contribution of the α- and β-isoforms of ER and the AMPA and kainate subtypes of glutamate receptors.

After E_2 administration in immature rodents, an inhibition of LH secretion was commonly observed [53, 54] and impeded the use of variations in LH secretion to investigate indirectly hypothalamic effects. In addition, there is physiologically a developmental reduction in serum LH concentrations between PND 15 and VO, both basally and in response to GnRH [15, 16], so that decreased LH levels could result from either negative feedback effects or accelerated maturation or both. Therefore, hypothalamic-pituitary maturation was assessed through evaluation of GnRH secretion during explant incubation and study of LH response to GnRH after steroid or EDC administration in vivo. We elected to study GnRH secretion at 15 days using explants from female rats based on our previous studies showing that the frequency of pulsatile GnRH secretion in vitro was maximally stimulated by E_2 in such conditions and through a mechanism dependent on perinatal brain sexual differentiation [12]. The exposure of infantile female rats to E_2 or o,p′-DDT for 5 or 10 days was followed by a dose-dependent increase in GnRH pulse frequency and a decrease in serum LH levels on PND 15, suggesting a stimulation of hypothalamic maturation and a negative feedback inhibition or an early maturation of the pituitary secretion. A negative feedback component at the hypothalamic and/or pituitary level was supported by the reduction in the postcastration rise of serum LH levels that was reported after treatment of mature or neonatal rats with o,p′-DDT [11, 41]. However, no change in LH secretion basally and in response to GnRH was found in another study 6 wk after o,p′-DDT treatment between PNDs 1 and 10 [42]. The unchanged LH response to GnRH on PNDs 15 and 22 suggests that the result of the combined inhibitory and stimulatory effects at those stages is a steady state. Then, early VO could result from either a peripheral effect of E_2 or DDT or early pituitary-ovarian activity or both. A central component at the time of VO is suggested by the brisk reduction in LH response to GnRH that was observed in the treated animals. Subsequently, early first estrus possibly confirmed premature activity of the hypothalamic-pituitary-ovarian axis. Such a pathophysiologic mechanism could be consistent with an involvement of DDT in the sexual precocity occurring after migration in internationally adopted children [1, 2, 9]. In a recent Danish study of such children, it was shown that developmental changes in pituitary-ovarian hormone levels were observable before onset of puberty and supported a hypothalamic-pituitary mechanism of early puberty [55]. Since our report of an association between sexual precocity and the detection of p,p′-DDE in the serum of migrating girls [1], early menarche was found to occur after prenatal or postnatal exposure to DDE and/or DDT [56, 57]. However, others did not observe changes in menarcheal age after prenatal or postnatal exposure to DDE [58, 59], and postnatal treatment of female monkeys with methoxychlor resulted in delayed nipple development and menarche [60]. Further studies are warranted to clarify the possible role of EDC nature and parameters of exposure (age, dose, duration) in those discrepant effects. In rodents, discrepant observations were made as well, since pesticides such as methoxychlor and lindane resulted, respectively, in precocity and delay in the age at VO, but both

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insecticides caused disturbances of estrous cyclicity [61–63], and decreased serum LH levels were reported after lindane administration [61]. The GnRH neuron itself could be targeted by EDCs, since the phytoestrogen coumestrol caused inhibitory effects on GnRH transcript expression in GT1–7 GnRH-secreting neuronal cells through the β subtype of ER [64].

When E_2 was injected for PND 6–40, early VO followed by permanent estrus was observed. After E_2 treatment was stopped, first estrus appeared at a markedly delayed age. This could indicate a peripheral stimulatory effect of the steroid together with a central inhibition during exposure. A slight anorexigenic effect of E_2 was observed, consistent with studies previously reported by Ramirez and Sawyer [65] and by Ramirez [66], whereas o,p′-DDT caused a dramatic decrease in body weight, suggesting a possible toxic effect of the insecticide [45].

In summary, evidence that DDT could influence the infantile female hypothalamic pituitary maturation was provided through early developmental acceleration of the GnRH secretion in vitro and early reduction of LH response to GnRH in vivo. It was also demonstrated that this chemical caused a precocious onset of puberty in vivo when immature individuals were transiently exposed to DDT. Further studies will aim to delineate its mechanism of action and address whether the GnRH neurons or other cell structures are primary targets.

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